CHAPTER 36

Genetic Engineering of Fruit Flavors

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INTRODUCTION

A fruit is the ripened ovary of a flowering plant that contains the seeds. The primary biological function of a fleshy fruit is to facilitate seed dispersal. To do so, fruits have been selected during evolution for the manifestation of a number of characters, one of which is flavor. Fruit flavor affects animals' perception of a specific type of fruit. It is therefore important for the reproductive success of flowering plants. Fruit flavor has also played an important role in human culture. Indeed, fruit crops have been cultivated by humans for both their nutritional values and diverse flavors.

Human perception of fruit flavor is generally considered to be a combination of taste and aroma (Baldwin et al. 2000). There are five primary taste sensations: sweetness, sourness, saltiness, bitterness, and, recently discovered, umaminess, each of which is determined by the contact of the mouth and tongue with sugars, acids, several amino acids, nucleotides, and a number of bitter compounds (Kinnamonll and Margolskeea 1996). Aroma is determined by volatile compounds sensed by the olfactory system (Goff and Klee 2006). Because flavor is an important attribute affecting human selection and enjoyment of fruits, the genetic improvement of flavors of fresh fruits has been an important endeavor in agriculture. With the advent of molecular biology and plant transformation, much attention has been paid to using genetic engineering for fruit flavor improvement.

CHEMISTRY AND BIOCHEMISTRY OF FRUIT FLAVORS

Fruit flavors are the sum of the interaction between sugars, acids, lipids, and a blend of volatile compounds (Acree 1993). The content of sugars, mainly glucose and fructose, and its ratio to the content of acids, such as citric acid and malic acid, determine the sweetness of fruits. In contrast, the volatile compounds involved in

Handbook of Fruit and Vegetable Flavors, Edited by Y. H. Hui
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aroma determination are often much more complex. Many fruits produce a large number of volatile compounds. The fruits of fresh tomato (*Solanum lycopersicum*), for example, produce more than 400 different volatile constituents (Buttery and Ling 1993). These volatile components include acyclic, cyclic, and heterocyclic hydrocarbons, alcohols, phenols, ethers, aldehydes, ketones, carboxylic acids, esters, and lactones, as well as nitrogen, sulfur, and halogen-containing compounds (Buttery et al. 1987, 1988, 1990; Lewinsohn et al. 2001; Linforth et al. 1994; Maul et al. 1998; Petro-Turza 1986). The contribution of individual volatiles to human perception of fruit flavor is not equal. For some fruits, flavor is determined by a few dominant volatile compounds. Banana (*Musa acuminata* Colla) flavor, for example, is mainly determined by 3-methybutyl acetate (Berger 1991). For Polynesian pineapple (*Ananas comosus*), esters, lactones, furanoids, and sulfur compounds act as potent odor components (Tokitomo et al. 2005). In contrast, approximately 30 volatiles were determined to contribute significantly to the fruit flavor of fresh tomato (Buttery 1993; Buttery and Ling 1993).

Although chemically complex, the majority of flavor volatiles from fruits are synthesized from three major biochemical pathways: the terpenoid pathway, the lipid degradation pathway, and the phenylpropanoid/benzenoid pathway (Dudareva et al. 2006; Hoffmann et al. 2003; Walker and Croteau 2000; Wang et al. 2001). The terpenoids compose the largest class of plant secondary metabolites. All terpenoids are synthesized from the universal five carbon precursors, isopentenyl diphosphate, and its allylic isomer dimethylallyl diphosphate. Based on the number of five carbon units they contain, terpenoids can be grouped into hemiterpenes (C5), monoterpenes (C10), sesquiterpenes (C15), homoterpenes (C11 and C16), diterpenes (C20), triterpenes (C30), tetraterpenes (C40), and polyterpenes (>C40). Mainly monoterpenes and sesquiterpenes have been identified at varying levels in the flavor profiles of most fruits. Monoterpenes are derived from acetyl-CoA by the mevalonic acid pathway localized in the cytosol (McCaskill and Croteau 1995). Sesquiterpenes are formed from pyruvate and glyceraldehyde-3-phosphate via the methylerythritol phosphate pathway localized in plastids (Rodriguez-Concepcion and Boronat 2002). The enzymes that catalyze the formation of monoterpenes and sesquiterpenes from their corresponding immediate universal substrates, geranyl diphosphate and farnesyl diphosphate, respectively, are called terpene synthases (TPSs). A large number of genes encoding TPSs have been isolated and characterized from a variety of plant species (Chen et al. 2003; Tholl 2006). This provides an important repository of genes for the genetic engineering of fruit flavors. In addition to terpenes synthesized by the action of TPSs, some volatiles are formed from the degradation of carotenoids, which are usually red, orange, or yellow pigments synthesized from the terpenoid pathway. For example, carotenoid-derived volatile norisoprenoids are important flavor compounds of fresh tomato fruits (Simkin et al. 2004).

A large number of volatiles such as aldehydes, alcohols, and esters are formed from the lipid degradation pathway (Feussner and Wasternack 2002). Unsaturated fatty acids, especially linoleic acid (18:2) and linolenic acid (18:3), are formed from lipids by the action of acyl hydrolase enzymes. Unsaturated fatty acids undergo dioxygenation in a reaction catalyzed by lipoxygenases (LOXs). LOXs can catalyze the oxygenation of polyenoic fatty acids at C9 or C13 positions to produce two groups of compounds: the 9-hydroperoxy and the 13-hydroperoxy derivatives of polyenoic fatty acids. Two branches of the LOX pathway, the hydroperoxide lyase

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(HPL) branch and the allene oxide synthase (AOS) branch, are responsible for the formation of volatile compounds. In the HPL branch pathway, HPL catalyzes the oxidative cleavage of hydroperoxy fatty acids that leads to the formation of short-chain C6- or C9-volatile aldehydes and the corresponding C12- or C9-ω fatty acids. The C6-aldehyde products of HPLs can be further converted to their isomers by spontaneous rearrangement or by alkenal isomerases, or they can be reduced to alcohols by the action of alcohol dehydrogenases (ADHs) (Prestage et al. 1999). The AOS branch of the LOX pathway leads to the formation of jasmonic acid, an important defense signaling and plant hormone (Liechti and Farmer 2006). The methyl ester of jasmonic acid, methyl jasmonate, is a volatile compound. The conversion from jasmonic acid to methyl jasmonate is catalyzed from jasmonic acid methyltransferase (Seo et al. 2001). The alcohols produced from fatty acid degradation can be used as substrates to form esters catalyzed by alcohol acyltransferases (AATs) (Beekwilder et al. 2004).

Phenylpropanoid/benzenoid compounds are derived from L-phenylalanine (Phe). Shared with the lignin biosynthetic pathway, a variety of hydroxycinnamic acids, aldehydes, and alcohols are formed from *trans*-cinnamic acid, which is an immediate product from Phe by the action of phenylalanine ammonia lyase (PAL), via a series of hydroxylation and methylation reactions (Humphreys and Chapple 2002). Some of these intermediates could be converted to volatile compounds. For example, coniferyl acetate can be converted to eugenol by the action of eugenol synthase (Koeduka et al. 2006). Eugenol can be further modified by methylation to form methyl eugenol (Gang et al. 2002), which is also a volatile compound. Benzenoid compounds also originate from *trans*-cinnamic acid. As a side branch of the general phenylpropanoid pathway, benzoic acid biosynthesis first involves the shortening of *trans*-cinnamic side chain by a C2 unit. The subsequent biochemical pathways leading to benzoic acid has not been solved. The biosynthesis of C6-C2 volatile compounds, such as phenylacetaldehyde, does not go through *trans*-cinnamic acid (Boatright et al. 2004). One recent study suggested that phenylacetaldehyde is formed directly from Phe via an unusual combined decarboxylation–amine oxidation reaction catalyzed by phenylacetaldehyde synthase (Kaminaga et al. 2006). The phenylpropanoid/benzenoid pathway also provides the precursors of acyl-CoAs, which are important for volatile ester formation catalyzed by AATs. In addition to Phe, other amino acids, such as isoleucine (Dickinson et al. 2000), may also serve as precursors for plant volatile formation.

REGULATION OF FLAVOR BIOGENESIS

In addition to biochemical pathways, the site of flavor compound production is also important for fruit flavor formation. Generally, many volatile and nonvolatile aroma compounds are produced in fruit skin, while organic acids and sugars are produced in pulp. For example, the skin and pulp of Queen Anne's pocket melon (*Cucumis melo*) showed difference in flavor profiles. The levels of volatiles in skin were significantly higher than those in pulp (Aubert and Pitrat 2006). After being synthesized, some volatile compounds could be released from the fruit surface immediately. Others may accumulate in the fruit and will not be released until the split opening. Some flavor compounds are accumulated in glycosidically conjugated forms (Parada

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et al. 2000). (1S, 2S)-1-Phenylpropane-1,2-diol 2-O-beta-D-glucopyranoside and p-menth-4(8)-ene-1,2-diol 1-O-alpha-L-arabinopyranosyl-(1-6)-beta-D-glucopyranoside, for example, are immediate precursors of 1-phenylpropane-1,2-diol and p-menth-4(8)-ene-1,2-diol, respectively. The latter two are typical volatiles found in the fruit of cape gooseberry (*Physalis peruviana*) (Mayorga et al. 2001). In some fruits, the levels of bound compounds increase significantly with maturation (Aubert et al. 2003), suggesting that both the biogenesis and accumulation of such bound compounds play important roles in the determination of fruit flavors.

In general, fruit flavor releasing is a dynamic process. In the mature process of fruits, the volatile composition of fruits changes along with the different stages of maturity. At the fully mature stage, key flavor compounds increase significantly, correlating with skin color development (Menager et al. 2004). During the maturation of snake fruit (*Salacca edulis* Reinw.) Pondoh, the contents of sucrose, glucose, fructose, and volatile compounds change drastically. While the contents of glucose, fructose, and volatile compounds achieve their maximal levels at the end of maturation, the contents of sucrose decrease (Supriyadi et al. 2002). Storage can also have an effect on fruit flavor. Some aroma-related volatile components increase during storage, while others, especially esters, may decrease (Chen et al. 2006).

Ethylene plays an important role in fruit flavor biogenesis. Due to its regulatory role in fruit ripening, ethylene is recognized as a "ripening hormone" (Abeles et al. 1992). The factors that regulate the biosynthesis and action of ethylene may impact fruit flavor formation. 1-Methylcyclopropene (MCP) is an ethylene inhibitor. MCP-treated apples (*Malus domestica*) were found to retain more alcohols, aldehydes, and β-damascenone volatiles than untreated apples (Lurie et al. 2002). In transgenic apples in which the ethylene signaling pathway is suppressed, the composition and accumulation of sugars, acids, and the aldehydes and alcohol precursors of volatile esters were not affected. However, the synthesis of ester volatiles was significantly suppressed (Dandekari et al. 2004). In addition to plant hormones, stress factors such as high levels of CO_2 can also have an additive effect on fruit flavor formation (Perez and Sanz 2001).

GENETIC ENGINEERING OF FRUIT FLAVORS: CASE STUDIES

Selection for important agricultural traits, such as overall yield and disease resistance, often results in the loss of aroma and taste in fruits and horticultural crops (Galili et al. 2002). Restoration of appealing flavors in fruits can be realized through classical breeding. For example, the distinct aromatic fragrance of *Lycopersicon peruvianum* LA 1554 has been introduced into the cultivated tomato, *Lycopersicon esculentum* (Kamal et al. 2001). These two species are distantly related. Ovule selection and culture method were used to circumvent the strong breeding barrier. From this breeding study, a large BC1F1 population was developed. Among the plants that were self-compatible and yielded fruits, fruits of some of these selected plants displayed an enriched sweet aromatic flavor. In sensory evaluation, panel opinion on flavor desirability significantly favored the BC1F1 fruits of some selected plants over the cultivar "Momotaro," one of the best consumer-rated Japanese commercial tomato cultivars.

Recently, Sams and Pantalone (2007) utilized molecular breeding to develop and release the edible vegetable soybean (*Glycine max*) "NUTRIVEG Soy6407," which

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significantly exhibited superior flavor than commercial edamame soybean varieties based on 2 years of sensory panel data. Those researchers are now utilizing microarray analyses and analytical chemistry to identify specific flavor compounds in order to breed superior new varieties of better-tasting vegetable soybean.

Despite the above-described successful examples, classical breeding programs are often labor-intensive and time-consuming. In addition, the traits of fruit flavors are genetically complex and difficult to be quantified (Galili et al. 2002), making them difficult targets for conventional breeding. With the advent of molecular biology and plant transformation, people become interested in using genetic engineering to improve fruit flavors. Genetic engineering may reduce some of the drawbacks of classical breeding. For example, it can introduce a single gene at a time, making the progress less complex. In addition, genetic engineering can introduce foreign genes to produce exotic flavors.

During the last decade, a number of genetic engineering studies were conducted with an objective to alter fruit flavors (Aharoni et al. 2005). These studies either modified existing pathways (e.g., up- or downregulation of one specific gene or redirection of flux to a desirable compound by blockage of competing pathways) or introduced novel genes not existing in the host plant. Most of these studies used the cauliflower mosaic virus 35S promoter (35S promoter), a constitutive promoter, to drive the expression of the transgene. Others used fruit-specific promoters. In the following, we will describe case by case the genetic engineering studies of fruit flavors using tomato as a model (Table 36.1). These studies are categorized based on the target biochemical pathway to be manipulated.

Manipulating the Fatty Acid Degradation Pathway

A successful attempt to modify fruit flavor is through the introduction of a yeast Δ-9 desaturase gene into tomato. In transgenic tomato fruits, the levels of palmitoleic acid, 9, 12-hexadienoic acid, and linoleic acid increased, accompanied with a reduction in palmitic acid and stearic acid. Alteration of the profile of fatty acids was associated with changes in certain flavor compounds derived from fatty acids, most notably *cis*-3-hexenol, 1-hexanol, hexanal, and *cis*-3-hexenal. Some flavor compounds that are not derived from fatty acids, including 6-methyl-5-hepten-2-one and 2-isobutylthiazole, also showed increases in transgenic fruits (Wang et al. 2001).

LOX is another important target for genetic modification of the fatty acid degradation pathway. Five *LOX* genes (*TomloxA*, *TomloxB*, *TomloxC*, *TomloxD*, and *TomloxE*) have been identified in tomato. When the expression of *TomloxA* and *TomloxB* was suppressed in tomato fruit via an antisense strategy, no significant alterations in the production of the known tomato flavor volatiles were detected (Griffiths et al. 1999). In contrast, depletion of the expression of *TomloxC* via cosuppression or antisense inhibition led to major decreases in the flavor volatiles in both fruit and leaf (Chen et al. 2004), suggesting that *TomloxC* is the major *LOX* gene responsible for the production of fatty acid-derived volatiles in tomato fruits.

In another study, a cucumber (*Cucumis sativus*) *HPL* gene was introduced into tomato plants (Matsui et al. 2001). Though high activity of the introduced HPL could be detected in the fruits of transgenic tomatoes, the composition of volatile short-chain aldehydes and alcohols was little changed. The failure to alter volatile profiles in HPL transgenic fruits may be due to the high levels of endogenous HPL activity that are responsible for the formation of 9-hydroperoxides (Matsui et al. 2001).

TABLE 36.1. Genetic Engineering of Fruit Flavors in Tomato

Pathway to Be Altered	Transgene	Origin of Transgene	Means of Genetic Engineering	Promoter Used	Changes of Volatile Profile	References
Lipid degradation pathway	Acyl-CoA Δ-9 desaturase gene	Yeast	OE	35S	*cis*-3-Hexenol ↑, 1-hexanol ↑, hexanal ↑, *cis*-hexenal ↑, 6-methyl-5-hepten-2-one ↑, 2-isobutylthiazole ↑	Wang and others (1996)
Lipid degradation pathway	*LOX*	Tomato	OE, AS, co-suppression	35S	Hexanal ↓, hexenal ↓, hexenol ↓	Chen and others (2004)
Lipid degradation pathway	*ADH*	Tomato	OE	35S, PG	Hexanol ↑, (Z)-3-hexenol ↑	Speirs and others (1998)
Terpenoid pathway	*LIS*	*Clarkia breweri*	OE	E8	(S)-Linalool ↑, 8-hydroxylinalool ↑	Lewinsohn and others (2001)
Terpenoid pathway	*GES*	Basil	OE	PG	Geraniol ↑ and its derivatives ↑	Davidovich-Rikanati and others (2007)
Carotenoid degradation pathway	*CCD*	Tomato	AS	35S	β-Ionone ↓, pseudoionone ↓, geranylacetone ↓	Simkin and others (2004)
Phenylpropanoid/ benzenoid pathway	*AADC*	Tomato	OE	35S	Phenylacetaldehyde ↑, 2-phenylethanl↑,1-nitro-2-phenylathane ↑	Tieman and others (2006)

AADC, aromatic L-amino acid decarboxylase gene; *AAT*, alcohol acyltransferase gene; *ADH*, alcohol dehydrogenase gene; AS, antisense; *CCD*, carotenoid cleavage dioxygenase gene; E8, a tomato fruit-specific promoter; *GES*, geraniol synthase gene; *LIS*, *S*-linalool synthase gene; *LOX*, lipoxygenase gene; OE, overexpression; PG, a tomato polygalacturonase promoter that is fruit specific; 35S, cauliflower mosaic virus 35S promoter.

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The transformed tomatoes have also been obtained by altering the expression of *ADH* genes driven by either the 35S promoter or the tomato fruit-specific tomato polygalacturonase (PG) promoter. In the ripening transgenetic tomatoes, modified ADH levels were found to have an important role on the balance between some of the aldehydes and the corresponding alcohols associated with flavor production. These phenotypes were transmitted to second-generation plants. In a preliminary taste trial, fruits with increased ADH activity and higher levels of alcohols were perceived as having a more intense "ripe fruit" flavor (Speirs et al. 1998).

Manipulating the Terpenoid Pathway

Because of the importance of the terpenoid pathway, genetic engineering has been used to manipulate this pathway, which resulted in the generation of transgenic plants containing high concentrations of provitamin A (β-carotene), such as "Golden Rice" (*Oryza sativa*) (Ye et al. 2000). The study in tomato with a gene encoding *S*-linalool synthase from *Clarkia breweri* flowers represents the first attempt to manipulate fruit flavor via the modification of the terpenoid pathway. In this study, transgenic tomato plants were generated by the introduction of a heterologous *C. breweri S*-linalool synthase gene (*LIS*), driven by a tomato late-ripening-specific *E8* promoter. The transgenic tomato synthesized and accumulated *S*-linalool and 8-hydroxylinalool in ripening fruits, which are absent in the control fruit. This study also showed that no other phenotypic alterations were observed despite the difference in fruit volatiles (Lewinsohn et al. 2001).

The flavor and aroma of tomato has recently been modified through the introduction of another monoterpenoid biosynthetic gene, a geraniol synthase gene (*GES*) from basil (*Ocimum basilicum*) (Davidovich-Rikanati et al. 2007). Transgene *GES* was under the control of a tomato ripening-specific PG promoter. The expression of *GES* in ripening tomatoes caused marked changes in fruit volatiles. The levels of some existing volatiles, such as neral plus geranial, were sixfold higher in *GES*-expressing fruits than those in control fruits. Some volatiles, such as geraniol, nerol, citronellol, and citronellic acid, which are absent in control fruits, are present in high concentration in transgenic fruits. The increases in the levels of key volatiles resulted in a marked impact on the organoleptic attributes of tomato fruits. In a flavor panel study, >90% of the untrained panelists reported differences in their perception of the smell. A majority of the untrained taste panelists preferred the transgenic fruits over controls (Davidovich-Rikanati et al. 2007).

Manipulating the Carotenoid Degradation Pathway

Geranylacetone, β-ionone, and pseudoionone are among a group of related terpenoid flavor volatile compounds that are generally present at relatively low levels but possess strong effects on the overall human perception of fruit flavors (Simkin et al. 2004). Recent biological studies demonstrated that these volatiles are synthesized from the degradation of carotenoids, of which carotenoid cleavage dioxygenases (CCDs) play an essential role (Simkin et al. 2004). *LeCCD1B*, a *CCD* gene isolated from tomato, can cleave multiple carotenoid substrates at 9, 10 (9′, 10′) bonds to produce a C14 dialdehyde and two C13 cyclohexones depending on the substrate. Transgenic tomato was generated in which the *LeCCD1B* gene was

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constitutively expressed in a reverse orientation (antisense). Transgenic fruits did not show significant modification in the carotenoid content of fruit tissue. However, when the volatile profile of tomato fruits was analyzed, a ≥50% decrease in β-ionone (a β-carotene-derived C13 cyclohexone) and a ≥60% decrease in geranylacetone (a C13 acyclic product likely derived from a lycopene precursor) were observed in selected transgenic lines (Simkin et al. 2004).

Manipulating the Phenylpropanoid/Benzenoid Pathway

Phenylethanol is an important flavor compound important for the flavor of many foods and fruits (Tieman et al. 2006). A recent study in tomato showed that 2-phenylethanol is derived from Phe through the action of a small family of decarboxylases that can mediate that pathway's first step. In *in vitro* studies, these enzymes catalyze the conversion of Phe to phenethylamine and tyrosine to tyramine with tyrosine as the preferred substrate. In tomato fruits, however, the levels of Phe are much higher than those of tyrosine, indicating that Phe is the *in planta* substrate. When either *LeAADC1A* or *LeAADC2*, two tomato decarboxylase genes, was overexpressed in transgenic tomato plants, fruits exhibited 10-fold increases in the emission of the products of the pathway, including phenylethanol and phenylacetaldehyde. In contrast, reduction of *LeAADC2* expression using antisense significantly reduced emissions of these volatiles (Tieman et al. 2006).

CONSIDERATIONS FOR FUTURE STUDIES

Flavor is a valuable character of the fruits and has an important role in people's choice of fruits. The flavors of many fresh commercially produced fruits, such as tomato, are generally considered to be poor. It is therefore highly desirable to develop new fruits with better flavors. Because of the complex nature of the flavor trait, genetic engineering theoretically offers an ideal solution to improving fruit flavors. In reality, the impact of this technique on fruit flavor improvement thus far has been only limited (Simkin et al. 2004; Speirs et al. 1998). Much basic and applied research is still needed to achieve this goal.

Fruit flavors are often controlled by a complex mixture of plant metabolites. The molecular and biochemical mechanisms underlying the biosynthesis of these large numbers of compounds are still far from being fully elucidated. Many previous studies dealt with a single gene/enzyme at a time. If an overexpression strategy is used, the availability of appropriate substrates for the introduced enzyme will be critical for the development of an expected flavor. In this case, a thorough understanding of the metabolic pathway leading to the formation of a specific substrate will be important. Therefore, gene and pathway identification is a key area in the study of fruit flavor biogenesis. In addition to biosynthetic genes, the mechanisms that regulate the biosynthesis of flavor compounds from multiple pathways also need to be characterized. In light of the fact that a large number of compounds are involved in fruit flavor, it is important to use novel strategies for experimentation. Various novel genomic tools will be proved useful for elucidation of biosynthetic pathways and regulatory networks that govern flavor biogenesis. Identification of such key elements will be essential for success of genetic engineering.

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The alteration of one metabolic pathway can have profound impacts on other metabolic pathways. For example, successful enrichment of monoterpene flavor compounds in transgenic tomato was at the expense of reduced lycopene accumulation (Davidovich-Rikanati et al. 2007). Such pleiotropic effects need to be evaluated. As a complex trait, fruit flavor can be influenced by many environmental factors, such as light intensity, atmospheric CO_2 concentration, temperature, relative humidity, and nutrient status. A deeper understanding of the environmental effects on the formation of fruit flavors of transgenic fruits will be important for the commercial success of such novel fruits. Many previous studies of the genetic engineering of fruit flavors lack a component of sensory evaluation. We have to bear in mind that alteration of fruit flavor does not necessarily lead to flavor improvement. Sensory evaluations will also be critical for the successful development of commercial cultivars. In addition, public perception of genetically modified foods also needs to be taken into consideration.

We are at an exciting time of conducting plant biological research and genetic engineering. At this genomics era, systems biology that integrates transcriptome, proteome, and metabolome will provide novel knowledge on the biosynthetic pathways, enzymes, genes, and regulatory mechanisms that control fruit flavor formation. With such information and better tools of genetic engineering becoming available, researchers have promising avenues to pursue the improvement of fruit flavor.

ACKNOWLEDGMENTS

The research on the functional genomics of plant volatiles in Feng Chen's lab was partly supported by the Tennessee Agricultural Experimental Station at the University of Tennessee. We thank Jun Hu for his assistance at the early stage of manuscript preparation.

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